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Variation in the faecal shedding of *Salmonella* and *E. coli* O157:H7 in lactating dairy cattle and examination of *Salmonella* genotypes using pulsed-field gel electrophoresis

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ABSTRACT

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Aims: To examine the variability in faecal shedding of *Salmonella* and *Escherichia coli* O157:H7 in healthy lactating dairy cattle and to evaluate the genetic relatedness of *Salmonella* isolates.

Methods: Faecal samples were obtained from lactating Holstein dairy cattle on four commercial farms in the southwestern US. All farms were within an 8-km radius and were sampled in August 2001, January 2002 and August 2002 (60 cows per farm per sampling; $n = 720$ total samples). Samples were cultured for *E. coli* O157:H7 and *Salmonella* and a portion of the recovered *Salmonella* isolates were examined for genetic relatedness using pulsed-field gel electrophoresis (PFGE).

Results: Faecal shedding of *E. coli* O157:H7 and *Salmonella* varied considerably between farms and at the different sampling times. Large fluctuations in the percentage of positive animals were observed from summer to summer for both of these pathogens. Similarly, *Salmonella* serotype and serotype prevalence varied from farm to farm and within farm from one sampling time to another. Multiple *Salmonella* genotypes were detected for a number of serotypes and identical genotypes were found on different farms with one genotype of *Salmonella* Senftenberg identified on three of the four farms.

Significance and Impact of the Study: This study demonstrated the wide variability in pathogen shedding within and among dairy farms all located in a small geographical region and highlights the complexity of pathogen control at the farm level.

Keywords: dairy cattle, *E. coli* O157:H7, genotype, pulsed-field gel electrophoresis, *Salmonella*.

INTRODUCTION

Ruminants are natural reservoirs for *Salmonella* and *Escherichia coli* O157:H7 and typically appear nonsymptomatic while shedding these bacteria into the environment (Gansheroff and O'Brien 2000). Although shedding is intermittent and often difficult to detect, these pathogens have been isolated from dairy and beef cattle at all stages of production and appear to be fairly widespread throughout the bovine population (Fedorka-Cray *et al.* 1998; Hancock *et al.* 1998; Elder *et al.* 2000).

Previous research has indicated that faecal shedding of *E. coli* O157:H7 and *Salmonella* varies due to season and possibly geographical location (Hancock *et al.* 1997; Wells *et al.* 1998; Galland *et al.* 2001; Troutt *et al.* 2001). Spatial and temporal clustering of *Salmonella* has been reported (Sato *et al.* 2001), leading some to suggest that epidemiology

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may differ among *Salmonella* strains (Besser *et al.* 2000). However, it is important to note that these isolates were all obtained from sick, diarrhetic dairy cattle. Research conducted by our laboratory reported a wide diversity of *Salmonella* species in healthy, lactating dairy cattle and indicated that this population of animals is a significant source of potential contamination of the food chain and environment (Fitzgerald *et al.* 2002).

To examine the variation in faecal shedding of *Salmonella* and *E. coli* O157:H7 and changes in *Salmonella* serotype prevalence in healthy, lactating dairy cattle, we sampled four commercial farms in the southwestern US, all within an 8-km radius, on three successive dates approximately 6 months apart. Furthermore, we used pulse-field gel electrophoresis (PFGE) to compare the genetic relatedness of individual *Salmonella* isolates collected from these animals.

MATERIALS AND METHODS

Source and collection of faecal samples

Lactating Holstein cows were sampled on four large (2000–3000 cows) commercial dairies in the southwestern US. Faecal samples were collected from 60 cows (early lactation, <100 days in milk) on each farm in the morning of the same day in August 2001, January 2002 and August 2002. Cows were restrained in self-locking stanchions and approx. 30 g of faecal material was obtained via rectal palpation. Faecal samples were shipped on ice to the Food and Feed Safety Research Unit in College Station, TX, USA, for isolation of *E. coli* O157:H7 and *Salmonella*.

Bacterial culture and isolation

Escherichia coli O157:H7 was cultured as described previously (Elder *et al.* 2000). Briefly, 10 g of faeces was enriched in 90 ml of Gram-negative broth containing vancomycin, cefixime and cefsulodin for 6 h at 37°C. This was followed by immunomagnetic bead separation and enrichment using anti-*E. coli* O157:H7 antibody-labelled paramagnetic beads (Neogen Corp., Lansing, MI, USA). Fifty microlitres of the resulting suspension was then spread onto CHROMagar^{TM} (DRG International, Mountain Side, NJ, USA) plates containing potassium tellurite. Plates were incubated (18 h, 37°C) and three sorbitol-negative colonies exhibiting typical *E. coli* O157:H7 colony phenotype were selected from each plate. Selected colonies were cultured in 2 ml of MacConkey broth and tryptic soya broth (TSB) for 18 h at 37°C. Aliquots of the broth cultures were heat-killed (100°C, 5 min) and tested for reactivity with anti-*E. coli* O157 monoclonal antibody 13B3 and anti-H7 monoclonal antibody 2B7 by enzyme-linked immunoassay. Isolates identified as *E. coli* O157:H7 were stored in glycerol (10%, v/v) and TSB at −80°C.

Salmonella was cultured by enriching approx. 10 g of faecal material in 90 ml tetrathionate broth for 24 h at 37°C. Following incubation, 200 μl of the above enrichment was added to 5 ml Rappaport-Vassiliadis R10 broth and incubated for an additional 24 h at 42°C before spread plating on brilliant green agar (BGA) supplemented with novobiocin (25 μg ml^{-1}). Colonies exhibiting typical *Salmonella* morphology were confirmed biochemically using lysine and triple sugar iron agars. *Salmonella*-positive samples were confirmed by slide agglutination using SM-O antiserum poly A-I and V-I, and group C1 factors. *Salmonella* isolates were stored in glycerol (10%, v/v) and TSB at −80°C. Serotyping was performed by the National Veterinary Services Laboratory in Ames, IA, USA.

Pulsed-field gel electrophoresis

Salmonella isolates collected in August 2001 and January 2002 were analysed by PFGE as described previously (Hume *et al.* 2001). Briefly, *Salmonella* isolates were thawed and spread on BGA plates and incubated as above. A single colony from each plate was picked and incubated overnight in 10 ml of TSB. Cells were washed three times in phosphate-buffered saline (PBS) by centrifugation (8000 *g*) and maintained in a final suspension of 5 ml PBS. Washed cells were placed in a water bath (45°C) and mixed with equal volumes of 1·8% (w/v) low melting temperature agarose in PBS. Cells with agarose were transferred to disposable plug moulds for polymerization (4°C). Plugs were incubated (50°C, 72 h) in 20 ml of lysis buffer [1% (w/v) sodium lauryl sarcosine; 0·5 M EDTA, pH 9–9·3; and 0·2 mg ml^{-1} proteinase K] before washing twice (30 min, 4°C) in TE (10 mM Tris; pH 8·0; 1 mM EDTA). Plugs containing lysed cells were washed (3×; 1 h each) in 40 ml of cold TE containing 40 μl of phenylmethylsulphonyl fluoride (100 mM in isopropanol) and then washed three additional times (1 h) in cold TE. One quarter of each plug was incubated with *Xba*I restriction endonuclease. Conditions for PFGE were: initial switch time = 0·1 s; final switch time = 90 s; included angle = 120°; 6 V cm^{-1}; buffer temperature = 12°C; run time = 22 h. Genotypic relatedness was determined with Molecular Analysis Fingerprinting Software, version 1·6 (Bio-Rad Laboratories, Hercules, CA, USA).

RESULTS

The prevalence of *E. coli* O157:H7 and *Salmonella* in faecal samples obtained from lactating dairy cattle are presented by farm and sampling time in Table 1. *Escherichia coli* O157:H7 was isolated only in the summer sampling times with no

Table 1 Prevalence of *E. coli* O157:H7 and *Salmonella* isolated from faeces of lactating dairy cows at three seasonal sampling times*

		E. coli O157:H7		*Salmonella*	
Farm	Sampling time	*n*	%	*n*	%
A	August 2001	21	35	15	25
	January 2002	0	0	0	0
	August 2002	1	1·7	22	37
B	August 2001	1	1·7	18	30
	January 2002	0	0	1	1·7
	August 2002	4	6·7	37	62
C	August 2001	2	3·3	56	93
	January 2002	0	0	16	27
	August 2002	0	0	52	87
D	August 2001	17	28	9	15
	January 2002	0	0	1	1·7
	August 2002	6	10	55	92

*Sixty lactating cows sampled on each farm at each sampling time.

positive samples found on any farm in January. The percentage of cows shedding *E. coli* O157:H7 varied from farm to farm (ranging from 0 to 35%) and from summer to summer on the same farm (1·7–35%). *Salmonella* shedding also showed a seasonality pattern, with a much lower percentage of cows shedding at the winter sampling time. The one exception was farm C, where 27% of the cows were positive for *Salmonella* in the winter. Farm C also had the highest frequency of summer samples positive for *Salmonella* each year. Summer to summer variation within farm was greater for *Salmonella* shedding, particularly in farms B and C, with a two- and sixfold increase observed in the number of positive samples from the summer of 2001 to the summer of 2002 respectively.

A comparison of *Salmonella* serotype prevalence by farm and sampling time is presented in Table 2. Overall, a total of 22 different serotypes were identified with Montevideo, Mbandaka, Kentucky and Senftenberg serotypes the most prevalent. Differences in serotype prevalence due to sampling time were found among farms and when serotypes were pooled across farms. On farm A, *Salmonella* Newport and Give were the most prevalent serotypes in the summer of 2001; however, no *Salmonella* was isolated in the winter, and the following summer Senftenberg and Cubana were the predominant serotypes (only two Newport isolates and no Give isolates were found). On farm B, *Salmonella* Kentucky was the predominant serotype in the summer of 2001 but was not found in the winter or summer of 2002. The prevalence of *Salmonella* was much higher on farm C compared with the other farms and similar to the other farms, serotype prevalence changed with sampling time. In the summer of 2001, Mbandaka and Montevideo were the predominant serotypes, changing to Soerenga in the winter and to Cerro the following summer (although Soerenga and Montevideo were present in the summer of 2002). Farm D had numerous serotypes identified in the summer of 2001 while the following summer, serotype Montevideo was the most common isolate. When the serotype data were pooled across farms, Mbandaka, Montevideo and Kentucky were the most prevalent serotypes isolated in the summer of 2001; Soerenga was the predominant winter isolate; and in the summer of 2002, Montevideo and Senftenberg were the most prevalent serotypes.

Genotypes of selected *Salmonella* isolates as determined by PFGE are presented in Table 3. Multiple genotypes were detected for all serotypes where more than one isolate was examined with the exception of *Salmonella* Give, Meleagridis,

Table 2 Comparison of *Salmonella* serotype prevalence (no. of isolates and percentage of total) among four dairy farms and three seasonal sampling times

	Farm			
Sampling time	A	B	C	D
August 2001	Newport (6, 40%)	Kentucky (6, 33%)	Mbandaka (23, 41%)	Mbandaka (2, 22%)
	Give (6, 40%)	Senftenberg (4, 22%)	Montevideo (14, 25%)	Other (7serotypes; 7, 78%)
	Senftenberg (2, 13%)	Montevideo (3, 17%)	Kentucky (8, 14%)	
	Havana (1, 6%)		Other (6 serotypes; 10, 18%)	
January 2002	None	Montevideo (1, 100%)	Soerenga (12, 75%)	Montevideo (1, 100%)
			Senftenberg (2, 12%)	
			Cubana (1, 6%)	
			Minnesota (1, 6%)	
August 2002	Senftenberg (7, 35%)	Senftenberg (5, 25%)	Cerro (4, 20%)	Montevideo(16, 80%)
	Cubana (5, 23%)	Montevideo (5, 25%)	Soerenga (3, 15%)	Kentucky (2, 10%)
	Newport (2, 9%)	Mbandaka (3, 15%)	Montevideo (3, 15%)	Anatum (2, 10%)
	Meleagridis (2, 9%)	Cerro (3, 15%)	Senftenberg (3, 15%)	
	Other (4 serotypes; 4, 18%)	Other (3 serotypes; 4, 20%)	Other (7 serotypes; 7, 35%)	

Table 3 Genotypes of selected *Salmonella* isolates collected from lactating dairy cattle*

	Farm				Total†	
Serotype	A	B	C	D	Isolates	Genotypes
Anatum			2 (2)	1	3	3
Bredeny				1	1	1
Cerro				1	1	1
Typhimurium var. Copenhagen			1		1	1
Cubana		1	1	1	3	3
Give	1 (6)				6	1
Kentucky		3 (6)	6 (9)	1	16	6
Mbandaka		1	9 (24)	1 (2)	27	8
Meleagridis		1		1	2	1
Minnesota			1		1	1
Montevideo		3 (4)	4 (15)	3 (4)	23	7
Oranienberg			2 (2)		2	2
Newport	3 (5)				5	3
Senftenberg	1 (2)	2 (5)	2 (3)	1	11	4
Soerenga			2 (11)		11	2
3, 10:1, monophasic			2 (2)		2	2
4, 12:2, monophasic		1		1	2	2

*Number of genotypes on a farm for a given serotype. Values in parentheses indicate the number of isolates of a given serovar isolated on each farm.
†Total number of isolates and genotypes for each serotype on all farms.

Table 4 Genotypes of *Salmonella* serotypes identified on four dairy farms

Serotype	Genotype	No. of isolates	Farm of origin
Mbandaka	II	14	C
Mbandaka	II	2	D
Montevideo	I	11	C
Montevideo	I	2	B
Montevideo	III	1	B
Montevideo	III	2	C
Montevideo	IV	1	B
Montevideo	IV	1	D
Senftenberg	IV	2	A
Senftenberg	IV	2	B
Senftenberg	IV	2	C

Mbandaka (farm D only) and Senftenberg (farm A only). However, in the case of Senftenberg and Mbandaka, when more than two isolates were examined on the other farms, multiple genotypes were found. The most predominant serotypes (total isolates across farms) contained more different genotypes, approximately one different genotype for every three isolates. The one exception is serotype Soerenga, of which 11 isolates were examined and only two different genotypes found.

Identical *Salmonella* genotypes were often found on multiple farms (Table 4). One genotype of Mbandaka and one of Senftenberg were identified on 2 and 3 farms respectively. Only one serotype (Montevideo) had multiple genotypes (3) with each different genotype observed on multiple farms. Farm B was the only farm where all three Montevideo genotypes were identified. Genotype D of the serotype Senftenberg was the only genotype identified on more than two farms.

DISCUSSION

The dairy industry in the US has changed substantially within the past 20 years. Farm size and animal concentrations have increased (United States Department of Agriculture, National Agriculture Statistics Service 2002), creating new health and environmental concerns. Furthermore, cull dairy cattle contribute approx. 25% of all nonfed beef in the US, thus are an important vehicle for transmission of food-borne pathogens to humans. Research has suggested that almost all dairy farms will have cattle testing positive for *E. coli* O157:H7 if screened often enough (Hancock *et al.* 1997) and the National Animal Health Monitoring System Dairy '96 study reported 5·4% of milk cows shed *Salmonella* and 27·5% of dairy operations had at least one cow shedding *Salmonella* (Wells *et al.* 1991). In the present study, we found a wide range in the percentage of cows shedding *E. coli* O157:H7 (0–35%) and *Salmonella* (0–93%) depending on sampling time and farm. Previous research has similarly shown a high degree of variation in faecal shedding of *E. coli* O157:H7 and *Salmonella* in dairy cattle, with reports ranging from 0·9 to 14% (Besser *et al.* 1997; Hancock *et al.* 1997; Mechie *et al.* 1997; Wells *et al.* 1998) and 5·5 to 57% (Wells *et al.* 1998, 2001) respectively. However, sampling techniques and culture methods were not consistent in these studies and may have contributed to the observed differences. Huston *et al.* (2002a) reported a wide range of *Salmonella* prevalence (<1 to 97%) in Ohio dairy herds, similar to what we observed in the present study. In the present study, using identical sampling and culture techniques on multiple farms, we demonstrated the profound differences that may occur in the faecal shedding of *E. coli* O157:H7 and *Salmonella*, not only between farms but also within a single dairy operation. Reasons for the high degree of variation in pathogen shedding from farm to farm are unknown, but may be related to numerous factors involving farm management, genetics, and nutrition. Management factors (feed types, housing) and seasonal effects associated with pathogen shedding in dairy cattle have been examined (Jones *et al.* 1982; Mechie *et al.* 1997; Vaessen *et al.* 1998; Garber *et al.* 1999; Kabagambe *et al.* 2000) and shown to have limited effects on pathogen shedding. However, it is important to note that all four farms sampled

in this study were located within an approx. 5-mile radius of one another and cows were housed and fed under similar conditions. Dairy size has also been implicated as a factor affecting faecal shedding of *Salmonella*. Farms milking >400 cows had more *Salmonella*-positive animals (56·5%) than 100–399 cow dairies (38·5%), and dairies <100 cows (4·8%) (Wells *et al.* 1998). Research conducted in Ohio (Huston *et al.* 2002a) and New York (Warnick *et al.* 2003) dairy farms reported that herd size was the only factor conducive to *Salmonella* shedding in dairy cows. In the present study, which sampled dairies with greater than 2000 head, we found similar shedding percentages as those stated previously above for large dairy farms.

Within farm, we found a substantial difference in the prevalence of *E. coli* O157:H7 (farms A, D) and *Salmonella* (farms B, D) from summer to summer. The reasons for these differences are unknown. Seasonal differences have been reported for *E. coli* O157:H7 (Hancock *et al.* 1997; Mechie *et al.* 1997; Wells *et al.* 1998), with shedding more prevalent in the summer months. Others have reported seasonal differences in *Salmonella* shedding on dairy farms, but no overall pattern when multiple farms were examined (Huston *et al.* 2002b). We observed a seasonal effect, not only in *E. coli* O157:H7 but also in *Salmonella* shedding. However, this explains the winter/summer difference but not the summer to summer differences we observed. A partial explanation may involve *Salmonella* populations. Interestingly, the decrease in *E. coli* O157:H7 prevalence from the first summer to the second on farms A and D, was essentially mirrored with an increase in the prevalence of *Salmonella*. Farms B and C, which had a low prevalence of *E. coli* O157:H7 at all sampling times, had a high percentage of *Salmonella*-positive cows each summer. This is consistent with other observations we made, in that dairies with a high percentage of *Salmonella*-positive animals tend to have very few animals shedding *E. coli* O157:H7 (T.S. Edrington, unpublished data).

In the present study, we identified 22 different *Salmonella* serotypes with Montevideo, Senftenberg, Mbandaka and Kentucky being the most common. Others have reported high numbers of *Salmonella* Montevideo (Galland *et al.* 2001; Wells *et al.* 2001; Fitzgerald *et al.* 2002), Senftenberg (Fitzgerald *et al.* 2002) and Kentucky (Wells *et al.* 2001; Huston *et al.* 2002b) isolated from dairy cattle. The serotype Mbandaka was frequently isolated in this research; however, only a few researchers have reported this isolate in dairy cattle (Jones *et al.* 1982; Galland *et al.* 2001; Wells *et al.* 2001; Huston *et al.* 2002b). A high diversity of *Salmonella* serotypes on dairy farms has been reported by others (Wells *et al.* 2001). The high number of serotypes found in the present study may be a result of the relocation of cows to the expanding dairies in the southwestern US or the introduction of heifers from other off-farm sources. Additional routes of *Salmonella* introduction may include, but are not limited to, people, vehicles, feed, rodents and birds.

There was some similarity in serotype prevalence among farms as might be expected due to their close proximity to one another. However, sampling time had a marked effect on the species of *Salmonella* isolated. On each farm, the most frequently isolated serotype in the summer of 2001 was not found in the winter sampling, and isolated infrequently the following summer. This same trend was observed when serotype data was pooled across farms. Galland *et al.* (2001) also reported significant serotype diversity due to season in *Salmonella* isolated from cull dairy cows.

Others have shown evidence of geographical distribution differences for *Salmonella* Typhimurium DT104, suggesting that epidemiology may differ among *Salmonella* strains (Besser *et al.* 2000). Geographical differences have been noted for serotype diversity and also for season of highest prevalence (Galland *et al.* 2001; Troutt *et al.* 2001). Spatial and temporal clustering of *Salmonella* Typhimurium, Montevideo, and several other serotypes has been reported (Sato *et al.* 2001) in dairy cattle in California; however, these were all isolates obtained from diarrhetic cattle, not healthy cattle as in the present study. These same researchers reported *Salmonella* prevalence and serotype diversity were greater for samples collected in the western US vs other regions of the US and in the summer months compared with other locations and winter sampling (Galland *et al.* 2001; Troutt *et al.* 2001). This may in part explain the differences in our results and the existing literature concerning prevalence rates and predominant serotypes.

The use of PFGE allows for the examination of genetic relatedness among bacterial isolates by comparison of endonuclease restriction band patterns. Multiple genotypes were frequently observed among serotypes within farm and among the four farms sampled. Interestingly, identical *Salmonella* genotypes (Mbandaka, Senftenberg and Montevideo) were found on multiple farms with one identical Montevideo genotype found on three separate farms. Although farm B is the only closed-herd farm (no animals have entered the herd from outside sources since the mid-1980s), due to the close proximity of these four farms to one another, these results are not surprising. Furthermore, the introduction of new cattle into Ohio dairy herds was not associated with *Salmonella* shedding status (Huston *et al.* 2002a). In dairy herds closed for more than 3 years, environmental contamination along with passively or latently infected cattle were suspected in persistent *Salmonella* infections (Wray *et al.* 1989). Transfer of *Salmonella* genotypes from one farm to another could easily occur as mentioned earlier, via vehicles, birds, rodents, insects, wind, water, or other unknown sources of bacterial transmission. To our knowledge this the first research to use PFGE to

analyse *Salmonella* isolated from healthy dairy cattle. Murinda *et al.* (2002) examined *Salmonella* isolated from cull dairy cows and bulk tank milk and reported genetic similarity among isolates; however, in all but one case, the related isolates were cultured from the same farm, not multiple farms as in the present study.

The results of our research highlight the genotypic variation in *Salmonella* isolated from healthy dairy cattle and offers insight into the complexity of the population dynamics of this food-borne pathogen. Furthermore, this study demonstrates the variability of pathogen shedding in healthy dairy cattle and the potential for environmental and human food contamination. The intermittent shedding of food-borne pathogens at the animal and farm level contributes to the challenge of pathogen control during the production stage. However, reducing the amount of food-borne pathogens entering the abattoir could produce 'the most significant reductions in human exposures to the organism and therefore in related illnesses and deaths' (Hynes and Wachsmuth 2000). A thorough understanding of the population dynamics of *Salmonella* and *E. coli* O157:H7 at the farm level is crucial before implementation of pathogen reduction strategies can be expected to be successful.

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